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Opsonin-dependent and independent surface phagocytosis of *S. aureus* proceeds independently of complement and complement receptors

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SUMMARY

We examined the mechanism of surface phagocytosis of *Staphylococcus aureus* by human polymorphonuclear leucocytes (PMN). Surface phagocytosis of unopsonized bacteria occurred, but was significantly enhanced by the presence of serum. The serum requirement was low, and a maximal effect occurred with serum concentrations of 0·25–0·5%. The opsonic effect of serum was not removed by heat inactivation of complement but was adsorbed, at low serum concentrations, by protein A, indicating that opsonin-dependent surface phagocytosis requires IgG but not C3. The requirement of opsonin-dependent surface phagocytosis for IgG was demonstrated further with purified IgG preparations as the sole opsonin. Activation of PMN by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol myristate acetate (PMA) increased opsonin-independent surface phagocytosis by 47% and 66%, respectively, but had no effect on opsonin-dependent surface phagocytosis. Blockade of the PMN iC3b receptor (CR3), which has lectin-like properties, by a panel of monoclonal antibodies against the α- and β-chains of CR3 did not inhibit the surface phagocytosis of opsonized or unopsonized *S. aureus*, and one antibody (NIMP-R10) enhanced opsonin-independent surface phagocytosis. These results indicate that the mechanism of surface phagocytosis is quite different to that observed in suspension assays. Opsonin-independent surace phagocytosis occurs and is enhanced by PMN activation, opsonin-dependent surface phagocytosis is dependent on IgG and not complement, and neither opsonin-independent nor -dependent surface phagocytosis proceeds through CR3.

INTRODUCTION

Most *in vitro* studies of the interaction between human polymorphonuclear leucocytes (PMN) and bacterial pathogens are performed in suspension assays, and the requirement for opsonins in serum is well established (Wright & Douglas, 1903). Optimal phagocytosis generally requires the presence of specific immunoglobulin and deposition of fragments derived from C3, the third component of complement, on the bacterial surface. In these assays little or no phagocytosis or killing of unopsonized bacteria is observed (Verhoef, Peterson & Quie, 1977b; Verhoef *et al.*, 1977a). *In vivo*, however, it is likely that PMN encounter surface-adherent bacteria present, for example, within abcesses or on epithelial and endothelial surfaces. Early studies by Wood, Smith & Watson (1946) and Wood *et al.* (1951) defined this phenomenon of 'surface phagocytosis' and demonstrated that pulmonary clearance of *Streptococcus pneumoniae* could proceed without the requirement for opsonins. More recently opsonin-independent phagocytosis by PMN or alveolar macrophages of *Staphylococcus aureus* and *Pseudomonas aeruginosa* attached to plastic or endothelial cells has been described (Lee *et al.*, 1983, 1984; Vandenbrouke-Grauls, Thijssen & Verhoef, 1984, 1985). However, the mechanism by which opsonin-dependent and opsonin-independent surface phagocytosis occurs remains uncertain, both with respect to the opsonic requirements for opsonin-dependent surface phagocytosis and whether specific cell-surface receptors on phagocytic cells are required for opsonin-independent surface phagocytosis.

In this report we demonstrate that opsonin-dependent surface phagocytosis *S. aureus* requires IgG but not complement, that PMN activation with synthetic chemotactic peptides or phorbol esters enhances the surface phagocytosis of unopsonized but not opsonized bacteria, and that the type 3 complement receptor (CR3, iC3b receptor), which has an epitope with lectin-like properties (Rose, Cain & Lachmann, 1985), is not the receptor for surface phagocytosis of unopsonized micro-organisms.

Abbreviations: CR3, type 3 complement receptor; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GHBSS, Hank's balanced salt solution with 0·1% gelatin; HHS, human serum heated at 56° for 30 min; PBS, phosphate-buffered saline, pH 7·45; PHS, pooled human serum; PMA, phorbol myristate acetate; PMN, human polymorphonuclear leucocytes.

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MATERIALS AND METHODS

Bacteria

S. aureus Wood 46, a protein A-negative strain, was used for all experiments. Colonies were stored on a blood agar plate at 4° and subcultured monthly. Bacteria were grown overnight in 10 ml of Brain-Heart Infusion broth (Oxoid, Basingstoke, Hants, U.K.) containing 50 μl of (methyl-^{3}H) thymidine (specific activity 0·92 TBq/mmol, CEA, Siren, France), centrifuged and washed twice with phosphate-buffered saline (PBS) and resuspended in PBS to a final concentration of approximately 2×10^9 bacterial/ml by a spectrophotometric method.

Polymorphonuclear leucocytes

Human polymorphonuclear leucocytes (PMN) were prepared from heparinized blood of healthy donors by dextran sedimentation, hypotonic lysis of erythrocytes and centrifugation on Ficoll–Paque (Pharmacia, Uppsala, Sweden). PMN were washed in calcium- and magnesium-free Hank's balanced salt solution (Gibco, Grand Island, NY) with 0·1% gelatin (GHBSS-Ca^{2+}, Mg^{2+} free) and finally resuspended in GHBSS with calcium and magnesium to a final concentration of 5×10^6 PMN/ml. Purity and viability, the latter assessed by exclusion of trypan blue, were >96%. Assays were commenced within 1 hr of purification.

Serum

Serum from a group of healthy donors was collected and pooled (PHS) and stored in small aliquots at −70° prior to use. Serum was heated at 56° for 30 min to inactivate complement when required (HHS). Serum was depleted of immunoglobulin by passage over a protein A–Sepharose CL-4B column (Pharmacia). Briefly, 10 ml of serum made 5 mM ethylene diamine tetraacetic acid (EDTA) was passaged twice over a 10 ml column, then collected and dialysed against PBS. The protein content of the depleted serum was 64% of the starting serum, as assessed by the change in absorbance at 280 nm, and appropriate corrections were made to compensate for this dilution. Approximately 94% of the original IgG was removed by this chromatographic procedure, as assessed by an immunodiffusion assay.

Human immunoglobulin

Human IgG was isolated from serum by caprylic acid precipitation (Steinbuch & Audran, 1969). The supernatant was dialysed against PBS and applied to an IgG affinity column, prepared by coupling the IgG fraction of sheep anti-human IgG (Silenus, Hawthorn, Victoria) to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. IgG was eluted with 1 M acetic acid, 0·1 M glycine-HCl, pH 3·0, and then dialysed against PBS. IgG concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of $E^{1\%}_{1cm}$ of 14. A single precipitin line was observed when IgG was tested by Ouchterlony double-diffusion with antiserum to whole human serum, and no contaminating bands were detectable on SDS–polyacrylamide gel electrophoresis analysis.

Additionally, three highly purified commercial preparations of IgG for intravenous use were tested for opsonic activity against *S. aureus* in the surface phagocytosis assay—preparations A (Intragam^R, Commonwealth Serum Laboratories, Parkville, Victoria), B (Gammagard^R, Hyland-Travenol, Glendale, CA) and C (Sandoglobulin^R, Sandoz, East Hanover, NJ).

Surface phagocytosis assay

This was performed essentially as described by Lee *et al.* (1983). Briefly, 500 μl of PMN ($2{\cdot}5 \times 10^6$) were added in triplicate to approximately 6×10^7 bacteria adherent to the wells of a 24-well tissue culture plate (Costar, Cambridge, MA). GHBSS or opsonin (PHS or HHS) were added to a final concentration of 10%, and the plates were incubated for 60 min at 37° without shaking. The supernatant was then aspirated and collected and the remaining adherent bacteria and PMN, released by incubation with 1 ml of 0·5% trypsin (Difco, Detroit, MI) 0·2% EDTA in PBS for 15 min at 37°, were added to their respective vials. Following a further wash of the wells with 1 ml PBS, cell-associated and non-cell-associated bacteria were separated by two washes and centrifugation steps. Four millilitres of ACS II scintillant (Amersham, Arlington Heights, IL) was then added to the PMN + bacteria (A) or bacteria (B) pellets and following counting in a Beckman LS 3801 scintillation counter, percentage phagocytosis (mean of triplicates) was determined by the formula $A/A+B \times 100$.

Neutrophil activation

PMN were 'activated' by treatment with the synthetic chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma, St Louis, MO) or the phorbol ester, phorbol myristate acetate (PMA, Sigma). Following purification PMN were preincubated at 37° for 30 min with 5×10^{-7} M FMLP or 10 ng/ml PMA, then added to the surface phagocytosis assay. Control PMN were preincubated in GHBSS at 37°.

Monoclonal antibodies

The following monoclonal antibodies directed against the type 3 complement receptor (CR3, iC3b receptor) were used. OKM1 (Ortho Diagnostic, Raritan, NJ), OKM10 (kindly provided by Dr G. Goldstein, Ortho Research Laboratory, Raritan, NJ), NIMP-R10 (kindly provided by Dr A. Lopez, IMVS, Adelaide) and anti-MAC-1 (Sera Lab, Crawley Down, West Susex, U.K.) are directed against the MW 170,000 α-chain of CR3. A monoclonal antibody, QEH 7.3E8, directed against the MW 95,000 β-chain of CR3 was kindly provided by Dr G. Russ (Queen Elizabeth Hospital, Adelaide). The effect of these antibodies on surface phagocytosis was determined by preincubation of PMN with saturating concentrations of the antibodies for 30 min on ice prior to commencement of the surface phagocytosis assay. Control PMN were preincubated on ice in GHBSS.

Statistics

Means were compared by Student's *t*-test, with $P < 0{\cdot}05$ considered to be significant. Figures shown represent the mean ± SD of three separate experiments performed in triplicate, unless otherwise indicated.

RESULTS

Opsonic requirements for surface phagocytosis

The serum requirements for surface phagocytosis of *S. aureus*

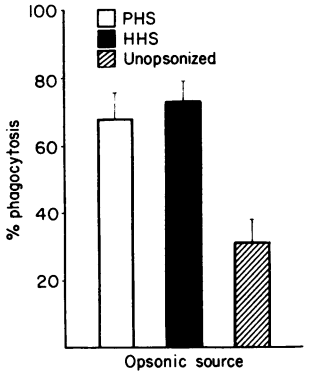

Figure 1. Surface phagocytosis of *S. aureus* in the presence of a final concentration of 10% pooled human serum, heat-inactivated serum, or in the absence of opsonins. The presence of PHS or HHS significantly enhanced surface phagocytosis when compared with no opsonin ($P < 0.005$).

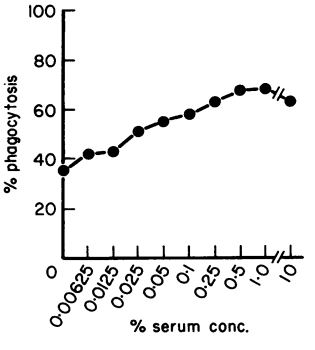

Figure 2. Effect of percentage serum concentration on the surface phagocytosis of *S. aureus* by human PMN.

Wood 46 strain are shown in Fig. 1. Although considerable phagocytic uptake occurred in the absence of opsonins, the presence of 10% serum substantially increased phagocytosis ($P < 0.005$). Both pooled human serum and heat-inactivated serum were equally effective in promoting phagocytosis, indicating that opsonins other than C3 are involved.

Figure 2 shows the percentage phagocytosis of *S. aureus* with different serum concentrations. The presence of minute serum concentrations (~0·01%) increased phagocytosis above that seen for unopsonized bacteria, and a maximal effect was observed with final serum concentrations of 0·25–0·5%. A direct effect of serum on PMN function was excluded as uptake of pre-opsonized and washed bacteria was comparable to that observed when serum was present in the phagocytic assay (data not shown).

Identification of IgG as the heat-stable opsonin for surface phagocytosis

Two approaches were used to determine the nature of the heat-stable opsonin in serum. Firstly, serum was passaged over a protein A affinity column to adsorb IgG, and secondly, the opsonic effect of purified IgG was examined.

Table 1. Effect of serum IgG depletion by protein A on surface phagocytosis of *S. aureus*

	% phagocytosis*	
Opsonin	Exp. 1	Exp. 2
None	38	29
Control serum†		
0·01%	45	38
0·1%	69	58
1·0%	78	67
IgG-depleted serum†		
0·01%	36	29
0·01%	48	36
1·0%	72	63

* Mean of triplicate determinations.

† Percentages shown represent the final serum concentration present in the assay during surface phagocytosis, and include a correction for dilution of the IgG-depleted serum during the protein A chromatographic step.

Table 2. Surface phagocytosis of *S. aureus*: opsonic effect of purified IgG*

Opsonin	% phagocytosis†
None ($n=3$)	34±6·2
Serum 1% ($n=3$)	74±5·3
Purified IgG ($n=3$)	65±3·5
Commercial IgG preparations ($n=2$)	
A	72±0·7
B	76±1·4
C	70±1·8

* All IgG preparations were used at final concentrations of 0·1 mg/ml.

† Mean±SD of two to three separate experiments performed in triplicate.

Initial experiments using protein A-adsorbed serum at a final concentration of 10% failed to show a reduction in surface phagocytosis compared with control serum. However, when the serum concentration was made limiting, IgG-depleted serum was less opsonic. Table 1 shows the results of two experiments in which the percentage phagocytosis observed with different concentrations of control and IgG-depleted serum was compared. The failure to detect an effect of protein A adsorption of serum when higher serum concentrations were tested probably reflects the inability of this procedure to completely adsorb all IgG from serum. In addition protein A affinity chromatography fails to deplete serum of the potentially highly opsonic IgG3 subclass (Van Loghem *et al.*, 1982).

In order to overcome the limitations of protein A adsorption of serum, IgG was purified from serum and examined for its

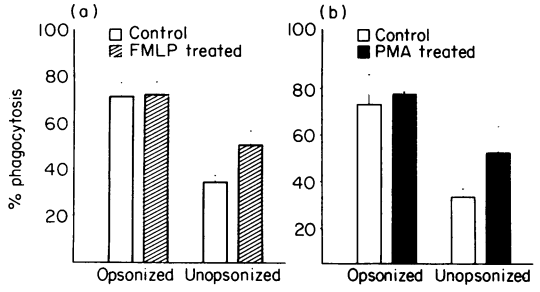

Figure 3. Effect of neutrophil activation on the opsonin-dependent and opsonin-independent surface phagocytosis of *S. aureus*. PMN were pretreated with 5×10^{-7}M FMLP (a) or 10 ng/ml PMA (b) prior to commencement of the surface phagocytosis assay. PMN activation significantly enhanced the opsonin-independent phagocytosis ($P=0{\cdot}016$ and $P=0{\cdot}027$, respectively).

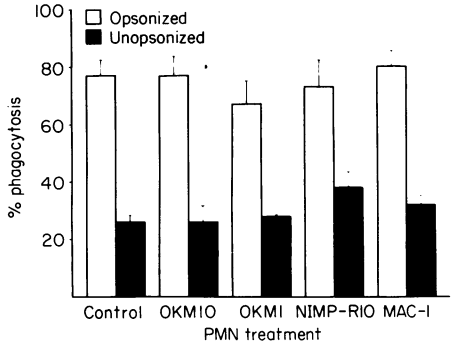

Figure 4. Effect of monoclonal antibodies against PMN CR3 α-chain on the surface phagocytosis of *S. aureus*. PMN were pretreated with saturating concentrations of monoclonal antibody prior to commencement of the surface phagocytosis assay. None of the antibodies blocked opsonin-dependent or opsonin-independent surface phagocytosis; NIMP-R10 significantly increased opsonin-independent phagocytosis ($P=0{\cdot}04$).

opsonic effect on the surface phagocytosis of *S. aureus*. Table 2 illustrates that affinity-purified IgG used at a final concentration at 0·1 mg/ml markedly enhanced phagocytosis ($P=0{\cdot}002$ when compared with no opsonin). In addition, three commercial immunoglobulin preparations available for intravenous use were also potent opsonins of surface phagocytosis. The extent of phagocytosis observed with the purified and commercial IgG preparations was similar to that seen with a serum concentration of 1%, containing approximately equivalent amounts of IgG.

Effects of neutrophil activation on surface phagocytosis

Exposure to PMN to chemotactic factors or phorbol esters results in neutrophil 'activation'. Activated neurophils show enhanced chemiluminescence and superoxide anion production in response to soluble and particulate stimuli and may have increased bactericidal and anti-helminthic activity (Issekutz, Lee & Biggar, 1979; Van Epps & Garcia, 1980; Moqbel *et al.*, 1987) as well as physiochemical changes including alterations in cell density, hydrophobicity and surface charge (Dahlgren & Stendahl, 1982; Moqbel *et al.*, 1987). Therefore, the effect of pretreatment of PMN with the synthetic chemotactic peptide FMLP or the phorbol ester PMA on surface phagocytosis was examined. Figure 3 demonstrates that FMLP and PMA pretreatment of PMN significantly enhanced the surface phagocytosis of unopsonized *S. aureus* by 47% and 66%, respectively ($P=0{\cdot}016$ and $P=0{\cdot}027$). In contrast, no effect on the surface phagocytosis of bacteria opsonized with PHS was detected.

Effect of anti-CR3 monoclonal antibodies on surface phagocytosis

CR3 has lectin-like properties and can bind to unopsonized yeast and zymosan at a site distinct from the iC3b-binding site (Ross *et al.*, 1985). CR3 is a member of a family of human leucocyte glycoproteins important in mediating adherence and effector functions related to cell contact (Sanchez-Madrid *et al.*, 1983). We have therefore investigated the possible role of CR3 in surface phagocytosis of unopsonized and opsonized *S. aureus* by examining the effect of preincubation of PMN with a variety of monoclonal antibodies directed against CR3. Figure 4 illustrates that preincubation of PMN with the monoclonal antibodies OKM10, OKM1, NIMP-R10 or anti-MAC-1, all of which are directed against the α-chain subunit of CR3, failed to inhibit the surface phagocytosis of either opsonized or unopsonized bacteria. Indeed, PMN pretreatment with NIMP-R10 resulted in significantly increased uptake of unopsonized *S. aureus* ($P=0{\cdot}04$). Additional studies with QEH 7.3E8, a monoclonal antibody directed against the β-chain subunit of CR3, also failed to inhibit surface phagocytosis of opsonized or unopsonized bacteria (data not shown).

DISCUSSION

In vivo it is likely that PMN frequently encounter pathogens attached to cell and tissue surfaces, rather than in suspension. This could occur when bacteria are attached to foreign bodies, to fibrin clots at sites of infection or to epithelial or endothelial surfaces. Unopsonized surface-adherent bacteria can be phagocytosed, and stimulate a respiratory burst in PMN (Lee *et al.*, 1983, 1984; Vandenbrouke-Grauls *et al.*, 1984, 1985; Hayashi, Lee & Quie, 1986). We have investigated the mechanisms involved in surface phagocytosis of bacteria, and have confirmed the above findings of surface phagocytosis of unopsonized bacteria. In addition we have defined the opsonic requirements for surface phagocytosis of opsonized *S. aureus*. Opsonin-dependent surface phagocytosis proceeds, unlike suspension phagocytosis, quite independently of complement, as both pooled and heat-inactivated serum were equally effective in promoting phagocytosis. An opsonic effect was observed with very low serum concentrations and was maximal at 0·25–0·5% PHS. Two lines of investigation identified the heat-stable opsonin in serum as IgG. At low concentrations, serum depleted of IgG by protein A chromatography was less opsonic than control serum. Higher serum concentrations of IgG-depleted serum ($\geqslant 1\%$) were, however, quite opsonic. This observation probably reflects the inability to completely adsorb all IgG with protein A treatment. In addition, IgG3 does not bind to protein A, and at least some of the opsonic activity seen with IgG-depleted serum could be attributable to remaining IgG3. Further evidence for the role of IgG in opsonin-dependent surface phagocytosis was obtained by using purified IgG as the

sole opsonin. Both affinity-purified and commercially available IgG were potent opsonins, resulting in phagocytic uptake comparable to that of 1% serum, containing a similar final concentration of IgG. These experiments clearly demonstrate that immunoglobulin, rather than a non-specific opsonin such as fibronectin, is required for opsonin-dependent surface phagocytosis. Vandenbroucke-Grauls, Thijssen & Verhoef (1987) have described an additional role of serum in surface phagocytosis of *S. aureus* on endothelial cells—opsonization not only enhances bacterial uptake but also protects bystander cells from damage by phagocytosing PMN.

Activation of PMN by chemotactic factors such as C5a or FMLP is associated with enhanced neutrophil function (Issekutz *et al.*, 1979; Van Epps & Garcia, 1980). Similar *in vitro* stimulation of PMN function is observed following treatment with lymphoblastoid cell supernatants or colony stimulating factors (Cross *et al.*, 1985; Lopez *et al.*, 1986). *In vivo*, PMN activation may provide a mechanism by which neutrophil function is modulated at sites of infection. We have observed a significant enhancement of opsonin-independent surface phagocytosis when PMN were activated by pretreatment with FMLP or PMA. No effect on opsonin-dependent surface phagocytosis was detected, possibly because near optimal phagocytosis was already occurring. The mechanism by which PMN activation results in enhanced opsonin-independent surface phagocytosis is unknown. Activation may be associated with increased hexose monophosphate shunt activity (Cross *et al.*, 1985), which appears to be required for phagocytosis of unopsonized bacteria (Vandenbroucke-Grauls *et al.*, 1984), or increased expression of a specific cell-surface receptor for opsonin-independent phagocytosis may be induced. Alternatively non-specific changes in size, density, hydrophobicity and surface charge, which may influence the PMN–bacteria interaction, may be responsible (Dahlgren & Stendahl, 1982; Moqbel *et al.*, 1987).

Neutrophil activation results in increased expression of CR3 (Berger *et al.*, 1984), a PMN receptor which appears to have two epitopes—one for iC3b and another with lectin-like properties which can bind beta-glucans on unopsonized yeast (Ross *et al.*, 1985; Ross *et al.*, 1987). As both enhanced opsonin-independent surface phagocytosis and CR3 up-regulation occurred together, we investigated the possibility that the non-iC3b binding site on CR3 represented a receptor for phagocytosis of unopsonized bacteria. Inhibition of CR3 by monoclonal antibodies against the α-chain or β-chain, common to the related leucocyte antigens LFA-1 and p150,95, failed to block surface phagocytosis. Preincubation with one monoclonal antibody, NIMP-R10 enhanced the phagocytosis of unopsonized *S. aureus*. This is analogous to the finding of Ding, Wright & Nathan (1987) who observed activation of mouse peritoneal macrophages by anti-MAC-1. It is possible that other cell-surface receptors distinct from CR3 but similarly up-regulated mediate opsonin-independent phagocytosis. Contributions from LFA-1 or p150,95 require exclusion with monoclonal antibodies to their α-chains. Parod, Godleski & Brain (1986) have characterized a MW 102,000 antigen on hamster pulmonary macrophages that may play a role in opsonin-independent phagocytosis.

In summary, in contrast to phagocytosis occurring in suspension, our studies indicate that opsonin-dependent surface phagocytosis *S. aureas* is dependent not on complement but solely on IgG, and that opsonin-independent phagocytosis is enhanced with PMN activation. Although PMN activation is associated with up-regulation of CR3, opsonin-independent surface phagocytosis does not proceed through this receptor. Further studies on the mechanism of this uptake are proceeding.

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